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Effects of Antrodia Camphorata on Viability, Apoptosis, and $[Ca^{2+}]_i$ in PC3 Human Prostate Cancer Cells

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Abstract

Antrodia camphorata (AC) has been used as a health supplement in Asia to control different cancers; however, the cellular mechanisms of its effects are unclear. The effect of AC on cultured human prostate cancer cells (PC3) has not been explored. This study examined the effect of AC on viability, apoptosis, mitogen-activated protein kinases (MAPKs) phosphorylation and Ca^{2+} handling in PC3 cells. AC at concentrations of 5-50 μg/ml did not affect cell viability, but at 100-200 μg/ml decreased viability and induced apoptosis in a concentration-dependent manner. AC at concentrations of 25-200 μg/ml did not alter basal $[Ca^{2+}]_i$, but at a concentration of 25 μg/ml decreased the $[Ca^{2+}]_i$ increases induced by ATP, bradykinin, histamine and thapsigargin. ATP, bradykinin and histamine increased cell viability whereas thapsigargin decreased it. AC (25 μg/ml) pretreatment inhibited ATP-, bradykinin-, and histamine-induced enhancement on viability, but reversed thapsigargin-induced cytotoxicity. Immunoblotting showed that AC (200 μg/ml) did not induce the phosphorylation of ERK, JNK, and p38 MAPKs. Collectively, in PC3 cells, AC exerted multiple effects on viability and $[Ca^{2+}]_i$, caused apoptosis *via* pathways unrelated to $[Ca^{2+}]_i$ signal and phosphorylation of ERK, JNK and p38 MAPKs.

Key Words: antrodia camphorata, apoptosis, Ca^{2+}, MAPKs, PC3, prostate cancer cells

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Introduction

Antrodia camphorata (AC) has been recently widely consumed as a health food in Taiwan and China (8). Many different effects of AC have been reported in *in vitro* and *in vivo* systems, although how AC exerts these diverse actions is unclear. These effects include anti-oxidation (34, 41), vasorelaxation (37), anti-inflammation (39), inhibition of apoptosis in PC12 cells (22), and induction of apoptosis in human hepatoma cells (15). The major active components of AC are polysaccharides (39), diterpenes (2), adenosine (22), zhankuic acids (32), and maleic/ succinic acid derivatives (26). The diverse effects of AC may be caused by the complexity of the active ingredients.

Ca^{2+} plays a pivotal second messenger role in many biological responses (33). An alteration in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) can result in different Ca^{2+}-coupled processes, such as secretion, contraction, photoreception, protein activation, fertilization, proliferation, and apoptosis (24). Another important effector in many cellular responses is the mitogen-activated protein kinases (MAPKs) (10, 11). MAPKs signaling cascades have been shown to be important in the differentiation, activation, proliferation, apoptosis, degranulation and migration of various cell types (28). There are three big families of MAPKs: ERK, JNK and p38 MAPKs (1); each of them plays specific roles in numerous cellular phenomena (3, 4). The effect of AC on $[Ca^{2+}]_i$ and MAPKs phosphorylation is unclear in any system.

Because AC has been shown to exert different effects on cancer and non-cancer cells from different origins, the current study was aimed to explore the effect of AC on the viability, apoptosis, $[Ca^{2+}]_i$, and MAPKs phosphorylation in human prostate cancer cells (PC3). The effect of AC on PC3 cells has not been examined. PC3 cells have been used as a model for prostate cancer research because they have properties similar to human prostate cancer cells (5, 6, 38). Many endogenous and exogenous agents can stimulate PC3 cells by causing a $[Ca^{2+}]_i$ rise, such as desipramine (16), safrole (7), estrogens (17), and histamine (20). The inositol-1,4,5-trisphosphate (IP3)-sensitive Ca^{2+} store is an important Ca^{2+} store that releases Ca^{2+} into the cytosol when cells are stimulated by endogenous agents such as estrogens (17) and histamine (20), but some exogenous agents can release Ca^{2+} from IP3-insensitive stores (7, 16). Furthermore, the Ca^{2+} release may induce Ca^{2+} influx across the plasma membrane *via* the process of store-operated Ca^{2+} entry (30). MAPKs also play a crucial role in the physiology of PC3 cells. Gopalakrishnan *et al*. (13) showed the modulation of activator protein-1 and MAPK pathway by flavonoids. JNK MAPKs were activated under the stimulation of phosphatases (18).

In this study, the effect of AC on viability and $[Ca^{2+}]_i$, under basal and agonists-stimulated conditions, the involvement of apoptosis and phosphorylation of ERK, JNK and p38 MAPKs were explored.

Materials and Methods

Chemicals

AC was a gift from Dintai Medical Co., Ltd. (1 F., No. 13, Lane 63, Yanji St., Kaohsiung 807, Taiwan). Air-dried AC mycelia powder samples were mixed with water and filtered through Whatman #1 paper four times and then air-dried. Mycelia powder was subsequently ground thoroughly and shaken with isotonic phosphate saline buffer (154 mM NaCl and 10 mM phosphate buffer, pH 7.4) at the ratio of 1:25 (w/v) at 25°C for 10 h, and then centrifuged at 3000 × g for 15 min, followed by filtering through a 0.2 μm pore size filter. The stock solution was stored at –20°C before experiments.

The reagents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/AM was from Molecular Probes (Eugene, OR, USA). Propidium iodide, and other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

PC3 cells obtained from American Type Culture Collection were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes, 5 mM glucose. The experimental reagents were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the solution used in experiments did not exceed 0.1%, and did not alter basal $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ Measurements

Trypsinized cells (10^6/ml) were loaded with 2 μM fura-2/AM for 30 min at 25°C in culture medium. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 (plus 5 mM $CaCl_2$) and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as previously described (14).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Augmentation in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at 10,000 cells/well in culture medium for 24 h in the presence of zero or different concentrations of AC. The cell viability detecting reagent WST-1 (4-[3-[4-lodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (10 μl pure solution) was added to samples after AC treatment, and cells were incubated for 30 min in a humidified atmosphere. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value. Experiments were repeated five times in six replicates.

Assessments of MAPKs by Immunoblotting

Cell concentrations were adjusted to 3×10^6 cells/dish and were seeded to 60 mm culture dishes. After 2 h of incubation, the culture medium was replaced by serum-free medium supplemented with 1 mg/ml bovine serum albumin (Gibco, Cleveland, OH, USA) and serum starvation was continued for 4 h, followed by the addition of 200 μg/ml AC for indicated time periods. The treatments were terminated by aspirating the supernatant and washing the dishes with a physiological saline. The cells were then lysed on ice for 5 min with 70 μl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). The lysed cells were scraped off the dish using a rubber policeman, transferred to microcentrifuge tubes, and vortexed for 10 sec. The cell lysates were then centrifuged to remove insoluble materials and the protein concentration of each sample was measured. Approximately 50 μg of supernatant protein from each sample was used for gel electrophoresis analysis on a 10% SDS-polyacrylamide gel. After electrophoresis, the fractionated proteins on gel were transferred to PVDF membranes (NEN™ Life Science Products, Inc., Boston, MA, USA). For immunoblotting, the membranes were blocked with 5% non-fat milk in TBST (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20) and incubated overnight with the primary antibody (rabbit anti-human phospho-ERK antibody, rabbit anti-human ERK antibody, rabbit anti-human phospho-JNK antibody, rabbit anti-human JNK antibody, rabbit anti-human phospho-p38 MAPK antibody, rabbit anti-human p38 MAPK antibody; all from Cell Signaling Technology, Beverly, MA, USA). Then the membranes were extensively washed with TBST and incubated for 60 min with the secondary antibody (goat anti-rabbit antibody, Transduction Laboratories, Lexington, KY, USA). After extensive washing with TBST, the immune complexes were detected by chemiluminescence using the Renaissance™ Western Blot Chemiluminescence Reagent Plus kit (NEN™ Life Science Products, Inc., Boston, MA, USA).

Measurements of Subdiploidy Nuclei by Flow Cytometry

After treatment with various concentrations of AC overnight, cells were collected from the media, and were washed with ice-cold physiological saline twice and resuspended in 3 ml of 70% ethanol. Then cells were suspended in 70% ethanol and stored at -20°C. The ethanol-suspended cells were centrifuged for 5 min at 200 × g. Ethanol was decanted thoroughly and the cell pellet was washed with ice-cold saline twice, and was then suspended in 1 ml propidium iodide (PI) solution (1% Triton X-100, 20 μg PI, 0.1 mg/ml RNAse). The cell pellet was incubated in the dark for 30 min at room temperature. Cell fluorescence was measured in the FACScan flow cytometer (Becton Dickinson immunocytometry systems, San Jose, CA, USA) and the data were analyzed using the MODFIT software.

Statistics

Data were reported as means ± SEM of five experiments and were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significant difference) procedure. A *P*-value less than 0.05 was considered significant.

Results

Effect of AC on Cell Viability

PC3 cells were cultured in the presence of 0-200 μg/ml AC and cell viability assays were performed.

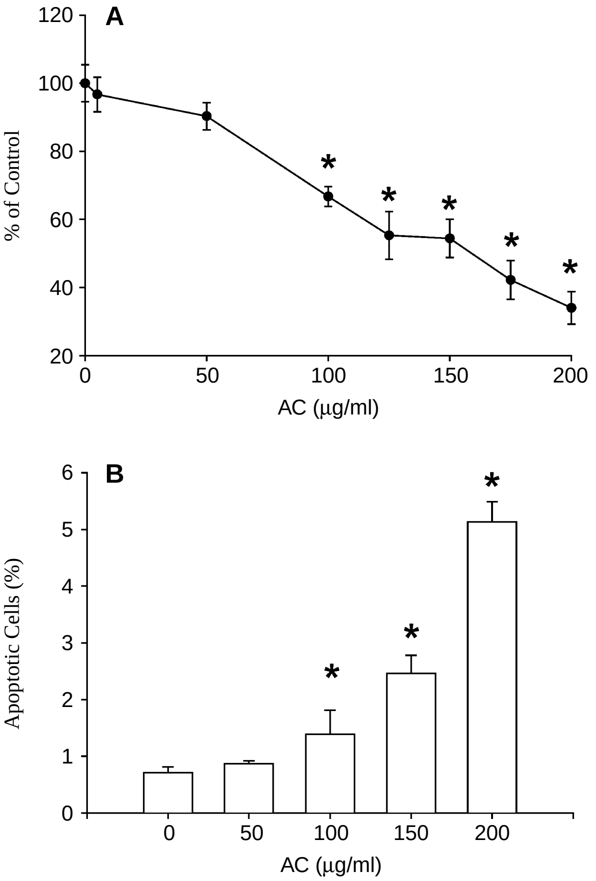

Fig. 1. Concentration-dependent effect of AC on viability and apoptosis of PC3 cells. Assays of viability and apoptosis are described in Materials and Methods. Data are presented as mean ± SEM of five experiments. *$P < 0.05$ compared with control.

Fig. 1A shows that 5-50 μg/ml AC did not alter viability; but 100-200 μg/ml AC decreased viability in a concentration-dependent manner ($P < 0.05$; n = 5).

Involvement of Apoptosis in AC-Induced Cytotoxicity

To investigate the characteristics of cell death observed in PC3 cells, we explored whether apoptosis occurred during AC incubation by measuring the increase in subdiploid peak. The data were presented as the percentage of apoptotic cells. As shown in Fig. 1B, apoptosis occurred in cells treated with 100-200 μg/ml AC in a concentration-dependent manner ($P < 0.05$; n = 5).

Effect of AC on Ca^{2+} Signaling

In order to understand the mechanisms of AC-induced apoptosis, efforts were made to examine the effect of AC on $[Ca^{2+}]_i$. It was found that AC (25-200 μg/ml) failed to induce a $[Ca^{2+}]_i$ increase (n = 5; not shown). Efforts were extended to explore the effect of AC on the $[Ca^{2+}]_i$ increases induced by four common Ca^{2+} mobilizers. Fig. 2A shows that basal $[Ca^{2+}]_i$ was 51 ±3 nM (n = 5). Addition of ATP (10 μM) induced an immediate $[Ca^{2+}]_i$ increase followed by a gradual decline. The maximum $[Ca^{2+}]_i$ was 65 ± 1 nM over baseline. After cells were pretreated with 25 μg/ml AC for 2 min, addition of ATP induced a $[Ca^{2+}]_i$ increase of 50 ±1 nM (n = 5). The area under the response-time curve between 25 and 150 sec was 23% less for AC+ATP compared with the ATP alone control ($P < 0.05$). Fig. 2B shows that addition of bradykinin (10 nM) induced a rise in $[Ca^{2+}]_i$. The peak $[Ca^{2+}]_i$ was 6 ± 1 nM over baseline. After cells were pretreated with 25 μg/ml AC for 2 min, addition of bradykinin failed to induce a $[Ca^{2+}]_i$ increase (n = 5). Fig. 2C shows that addition of histamine (10 μM) induced an immediate $[Ca^{2+}]_i$ increase followed by a gradual decline. The peak $[Ca^{2+}]_i$ was 25 ± 1 nM over baseline. After cells were pretreated with 25 μg/ml AC for 2 min, addition of histamine induced a $[Ca^{2+}]_i$ increase with a peak of 20 ± 1 nM (n = 5). The area under the response-time curve was 31% less for AC+histamine compared with the histamine alone control ($P < 0.05$). We next examined the effect of a different type of Ca^{2+} mobilizer: thapsigargin, an exogenous compound that increased $[Ca^{2+}]_i$ *via* inhibition of endoplasmic reticulum Ca^{2+} pumps (36). Fig. 2D shows that addition of thapsigargin (1 μM) induced a gradual $[Ca^{2+}]_i$ increase that reached 23 ±1 nM over baseline at 150 sec. After cells were pretreated with 25 μg/ml AC for 2 min, addition of thapsigargin induced a smaller $[Ca^{2+}]_i$ increase with an area under the response-time curve 10% less for AC+thapsigargin compared with the thapsigargin alone control ($P < 0.05$).

Effect of AC on Agonists-Induced Alterations in Viability

Because AC inhibited the Ca^{2+} signal induced by ATP, bradykinin, histamine and thapsigargin, the interaction of AC and these four agonists on viability was explored. Table 1 shows that, overnight incubation with 10 μM ATP induced an increase in viability by 20.1 ± 0.2% (n = 5; $P < 0.05$). Incubation with 25 μg/ml AC alone did not significantly alter viability but decreased ATP-induced increase in viability by 17.7% (n = 5; $P < 0.05$). Incubation with bradykinin (1 μM) alone increased viability by 36.2 ±2.5% (n = 5; $P < 0.05$). AC pretreatment decreased bradykinin-induced increase in viability by 25.7% (n = 5; $P < 0.05$). Histamine (10 μM) increased viability by 32.3 ± 0.06% (n = 5; $P < 0.05$). AC pretreatment decreased the effect of histamine by 25.8%. Incubation with thapsigargin (1 μM) decreased viability to 91.6

Table 1. Effect of AC on ATP, bradykinin, histamine, and thapsigargin-induced alterations in viability. Data are mean ±SEM of five experiments. * $P < 0.05$ compared with control. #: AC significantly ($P < 0.05$) enhanced ATP, bradykinin and histamine-induced effect on viability.

	Viability (%)
control	100
AC (25 μg/ml)	102.1 ±1.3
ATP (10 μM)	120.1 ±0.2*
AC+ATP	102.4 ±2.2 #
Bradykinin (1 μM)	136.2 ±2.5*
AC+Bradykinin	110.5 ±3.1 #
Histamine (10 μM)	132.3 ±0.1*
AC+Histamine	106.5 ±1.32 #
Thapsigargin (1 μM)	91.6 ±2.1*
AC+Thapsigargin	105.3 ±3.0 #

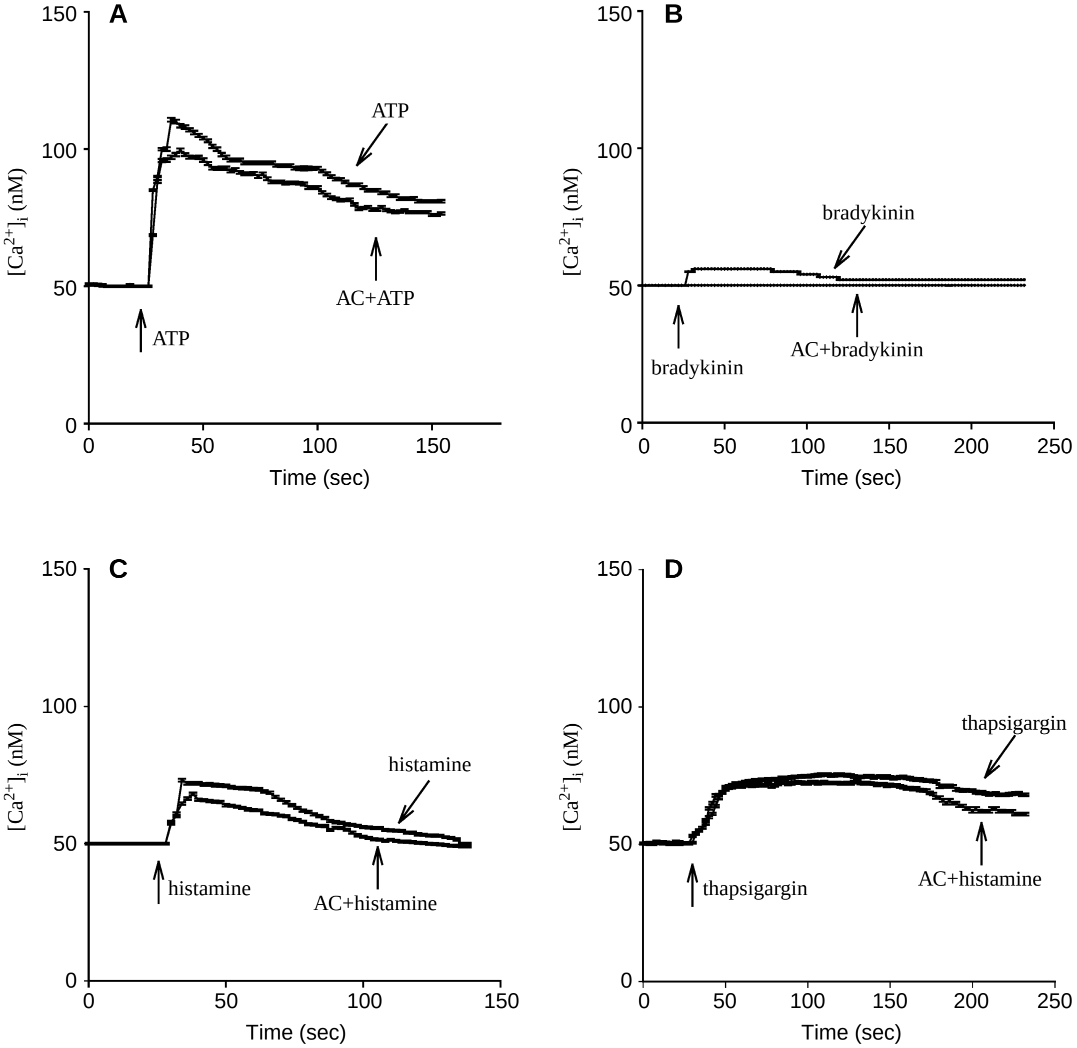

Fig. 2. Effects of AC on (A) 10 μM ATP, (B) 1 μM bradykinin, (C) 10 μM histamine and (D) 1 μM thapsigargin-induced increases in $[Ca^{2+}]_i$. AC (25 μg/ml) was added 150 sec before the agonists (not shown). Data are mean ±SEM of five experiments.

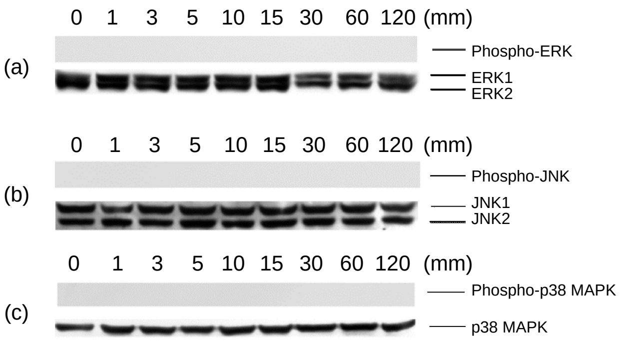

Fig. 3. No effect of AC on the phosphorylation of ERK, JNK and p38 MAPKs. Cells were treated with 200 μg/ml AC for indicated time periods. ERK, JNK, and p38 were detected in immunoblots using antibodies specific for each kinase. Phospho-ERK, phospho-JNK and phospho-p38 were not detected. Data are typical of five experiments.

±2.11% (n = 5; $P < 0.05$). AC completely prevented thapsigargin-induced cell death (n = 5; $P < 0.05$).

No Effects of AC on Phosphorylation of MAPKs

Previous studies have shown that activation of MAPKs is related to apoptosis (35, 40). Thus experiments were performed to explore whether AC altered the phosphorylation of ERK, JNK and p38. Fig. 3 shows that the expression of phosphorylated ERK, JNK, and p38 was not detected (n = 5).

Discussion

Despite the various presumable effects of AC in controlling or treating different health problems, the underlying cellular mechanisms of this Chinese medicine is unclear. This study was the first to explore the effect of AC on human prostate cancer cells. The data suggest that AC induced concentration-dependent cytotoxicity which involved apoptosis. AC was shown to prevent PC12 cells from apoptosis (22), but, on the other hand, it induced apoptosis in human hepatoma cells (15). Thus it appears that AC acts on proliferation differently in different cell types.

In some cases, apoptosis was caused by a preceding rise in $[Ca^{2+}]_i$. Priceman *et al.* (29) showed that Ca^{2+}-dependent upregulation of E4BP4 expression correlated with glucocorticoid-evoked apoptosis of human leukemic CEM cells. In DU145 prostate cancer cells, Savino *et al.* (31) showed that a $[Ca^{2+}]_i$ rise was required for diindolylmethane-induced apoptosis. Conversely, apoptosis could be triggered in the absence of a change in $[Ca^{2+}]_i$ in some cell types such as thymic lymphoma cells (23), neutrophils (9), and pancreatic islet cells (1), *etc*. We found that AC at concentrations that induced apoptosis did not cause $[Ca^{2+}]_i$ rises. Thus it seems that AC induces apoptosis *via* Ca^{2+}-independent pathways. While AC at a low concentration (25 μg/ml) did not alter basal $[Ca^{2+}]_i$, acute pretreatment (120 sec) with AC inhibited the $[Ca^{2+}]_i$ increases induced by ATP, bradykinin, histamine, and thapsigargin. These four ligands increase $[Ca^{2+}]_i$ *via* different mechanisms. ATP, bradykinin and histamine act by stimulating G-protein coupled receptors on plasma membrane; whereas thapsigargin acts by crossing plasma membrane and inhibiting the endoplasmic reticulum ATP pump. Because AC nonselectively inhibited the Ca^{2+} signal evoked by four different ligands, it appears that AC might inhibit Ca^{2+} movement in general.

Although AC inhibited the Ca^{2+} signals induced by the four ligands, it affected the effects of these ligands on viability differently. ATP, bradykinin and histamine all increased cell viability whereas thapsigargin decreased it. AC (25 μg/ml) pretreatment appeared to protect cells from the stimulatory or inhibitory effects of these ligands. Thus even at higher concentrations (150-200 μM) AC were cytotoxic to PC3 cells, at a non-cytotoxic concentration of 25 μg/ml, AC could protect cells.

In addition to Ca^{2+}, MAPKs are thought to play a key role in triggering apoptosis (12, 25). However, AC failed to induce the phosphorylation of ERK, JNK and p38 MAPKS, the three representative groups of MAPKs (19, 21, 27). Thus, mechanisms other than Ca^{2+} signaling and phosphorylation of ERK, JNK and p38 MAPKs are responsible for AC-induced apoptosis.

Together, we have demonstrated that in PC3 cells, AC exerted multiple effects on viability and $[Ca^{2+}]_i$, and evoked apoptosis *via* pathways independent of $[Ca^{2+}]_i$ and phosphorylation of MAPKs.

Acknowledgments

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